THE INHIBITIVE ACTION OF BILE UPON B. COLI.*

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Media containing bile or bile salts have come to be widely used for the isolation of *B. coli* from sewage and polluted water. Bile media have also been used extensively for the presumptive test. In the second edition of *Standard Methods of Water Analysis*,[1] lactose bile medium is recommended for the quantitative estimation of the *B. coli* group.

There is no doubt that the use of a bile medium offers certain advantages. Gas formation in a lactose bile fermentation tube is more likely to denote the presence of *B. coli* than gas formation in a dextrose broth fermentation tube. It is also true that in comparative series, lactose bile tubes are not so likely to show gas formation as plain lactose broth. These facts lessen the labor of plating as well as the thankless task of establishing the relation of gas-producing organisms to the *B. coli* group.

On the other side is a disadvantage not so generally recognized. This is the circumstance that *B. coli* itself is inhibited in a marked degree in media containing bile. It is true that Prescott and Winslow,[2] Longley and Baton,[3] and others have found that with some waters dextrose broth yields a larger proportion of positive results than lactose bile, but the use of different carbohydrates, lactose in one medium, dextrose in another, introduces a disturbing factor into such comparisons, and furthermore, the number of *B. coli* actually present in the water tested was an unknown factor. Ruediger,[4] working with polluted river water in North Dakota, has also noted inhibition in bile media as appears from the statement:

"It seems that less *B. coli* colonies will be found in the bile agar than in ordinary litmus lactose agar, although our work is not far enough advanced to draw positive conclusions."

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[1] American Public Health Association, 1912.

[2] *Rept. Am. Pub. Health Assn.*, 1907, 33(2), p. 128.

[3] *Jour. Infect. Dis.*, 1907, 4, p. 3.

[4] *Jour. Am. Pub. Health Assn.*, 1911, 1, p. 831.

The extent to which bile inhibits pure cultures of *B. coli* seems not to have been determined. With a view to ascertaining the degree of inhibition, a number of pure cultures of various ages and histories were plated in suitable suspensions and in parallel series upon plain agar and bile agar.[1] The majority of these were saccharose-fermenting strains. The bile agar was prepared as follows:

LACTOSE BILE AGAR

1,000 c.c. fresh, neutral ox bile
10 gms. lactose
10 gms. Witte's peptone
15 gms. agar

The agar, peptone, and lactose are dissolved in bile without water, boiling as little as possible. When dissolved, the medium is filtered without titration, tubed, and sterlized in the autoclav 3 minutes at 15 pounds pressure.

The following table shows certain typical results.

TABLE 1.

COLONY COUNT
48 Hours at 37° C.

Strain		Plain Agar	Bile Agar
Fecal strains transferred on nutrient agar at 48-hr. intervals	1 (47th transfer)	390	126
	2 (204th ")	451+	314
	3 (208th ")	403	34
	4* (47th ")	450	120
	5† (47th ")	600	180
Suspensions in flasks of sterile tap water‡	6	141	128
	7	683	507
	8	22+	8
	9	268	125
Freshly isolated from urine and feces of different persons	10	125	46
	11	263	28
	12	226	25
	13	341	85
	14	820	22

* Culture incubated for 2 weeks at 37° C. before plating.

† Culture kept 2 weeks in ice-chest before plating.

‡ Water suspension of pure culture kept 1 year at room temperature.

It is thus apparent that both freshly isolated strains of *B. coli* and those under long cultivation are inhibited by bile to a noteworthy degree. Four strains suspended in flasks of tap water in the laboratory at room temperature for one year showed at least as large a proportion of bile-viable cells as did the same strains grown on agar with 2-day transfers during the same period. Several strains freshly isolated from human feces were inhibited to a some-

[1] I have been assisted in this work by Mr. C. C. Hommon and Miss Edith Prindeville.

what greater degree on the average than other strains grown for several scores of generations on nutrient agar. It was noticed from time to time that different strains did not behave exactly alike in their resistance to bile and that different lots of ox bile varied in restraining power.

In the use of bile media in sanitary water analysis, it has been assumed, first, that the inhibitory effect of bile upon *B. coli* was slight, second, that the *B. coli* cells that are unable to grow in bile media are those that have been "attenuated" by a long sojourn in water and are hence of little significance. The following sentences from the second edition of *Standard Methods of Water Analysis* deal with this question:

"Attenuated *B. coli* does not represent recent contamination and all *B. coli* not attenuated grows readily in lactose bile" (p. 87).

"After numerous experiments it has been found that the lactose bile medium is slightly inhibitive to *B. coli* especially in attenuated form so that any positive tests with this medium indicate recent or fresh contamination" (p. 88).

"In the interpretation of the sanitary quality of the water, it is best to discount the presence of attenuated *B. coli* and to be sure to obtain all vigorous types. The lactose bile medium accomplishes both of these objects" (p. 91).

The belief that inability to grow in bile media is increased by prolonged suspension in water seems to be contradicted by the observations recorded above. Whether the inhibition shown in Table 1 is slight, is a matter of opinion.

With a view to testing the question of "attenuation" still further, 100 colonies of *B. coli* were fished from pure culture plates on plain agar and the same number from bile agar plates. The conditions were made absolutely parallel in all cases. If it were true that the more "vigorous" cells grew only on bile agar, one might, perhaps, expect more vigorous manifestations of physiological activity from the bile agar colonies than from those off the plain agar. Tested for milk coagulation (48 hours) and maximum indol production (4 days), the following results were obtained:

	Total Number of *B. coli* Fished	Milk Coagulation (48 Hours)	Maximum Indol Production (4 Days)
Plain agar	100	75	41
Bile agar	100	73	37

So far as these observations go, therefore, the cells that grow on bile agar are no more "vigorous" as regards fermentative and proteolytic power than the cells growing on plain agar. It is possible to assume of course that some of the cells growing on bile agar have been affected by the inhibiting agent in such a way that they have been reduced in their biological qualities to the level of the "weaker" cells growing on plain agar, but such an assumption puts the question beyond the range of experimentation.

It is impossible for obvious reasons to apply to methods of water examination the results obtained with pure cultures without further testing. Bile media have been used in water analysis in two somewhat different ways: (1) as "enrichment" media where the water is incubated, for example, in lactose bile and plated out after 24 to 48 hours' growth; this preliminary step is followed by definite identification of *B. coli;* (2) as "presumptive tests" where a given amount of gas (25 per cent after 72 hours' incubation—Jackson) is considered to be positive evidence of the presence of *B. coli.* Two questions, related but distinct, arise in this connection: first, to what extent we are justified in assuming that *B. coli* is present in lactose bile tubes yielding more than 25 per cent of gas; second, whether the *B. coli* cells surviving incubation in bile media—either in "preliminary enrichment" or in "presumptive tests"—fairly represent the number of colon bacilli originally present in the water.

There is little if any doubt that nearly all lactose bile tubes, giving more than 25 per cent of gas after 72 hours, contain *B. coli,* as has been shown by Jackson,[1] Prescott and Winslow,[2] and others. Frost[3] found that *B. coli* could be demonstrated in over 90 per cent of lactose bile tubes showing 20 per cent or more of gas. My own experience (Tables 3 and 4) practically coincides with that of Frost. This simply means, however, that other gas-producing bacteria are inhibited in larger measure than *B. coli* and indicates nothing as to the extent to which *B. coli* cells themselves refuse to grow in lactose bile. Some tests were made therefore with samples of water and sewage, unusual pains being taken to isolate *B. coli* from

[1] *Jour. Infect. Dis.*, Suppl. No. 3, 1907, p. 33.

[2] *Rept. Am. Pub. Health Assn.*, 1907, 33 (2) p. 128.

[3] *Hyg. Lab. Bull.*, No. 78, p. 134. Washington, 1911.

every sample giving the standard indications. In the observations here set down, ox bile freshly obtained from the Union Stockyards in Chicago has been used. The media have been prepared in accordance with the second edition of *Standard Methods of Water Analysis* (Am. Pub. Health Assn., 1912).

The isolation of *B. coli* from fermentation tubes was carried out by plating in litmus lactose agar from those tubes showing gas production after 24 hours' growth at 37° C. In case typical red colonies did not appear on the plate, a second plating was made after 48 hours, and a second negative result was followed up in most cases by a third plating after 72 hours. Nearly one-fourth of the tubes in which the plating gave negative results after 24 hours gave positive results by the 48 hour plating, but a third plating succeeding two negative results was rarely successful. Colonies were fished from the litmus lactose agar plates to Russell's medium,[1] and from this to gelatin. It is advisable to transfer several colonies from the agar plates even when the appearance of the plate is not encouraging, since, as is well-known, *B. coli* does not always produce typical colonies on litmus lactose agar. I have isolated *B. coli* a number of times from unpromising-looking plates. Nearly all cultures giving a characteristic reaction in Russell's medium turn out to be members of the *B. coli* group, but I have always controlled the results with gelatin-tube inoculation (14 days). About two-thirds of the cultures were also inoculated into milk, but since this procedure in no case modified the conclusions drawn from the behavior of the cultures in Russell's medium and in gelatin it was discontinued.

The following tables (Tables 2 and 3) show the results obtained with Lake Michigan water as drawn from the laboratory tap (1912). In all cases compared, the same sample of water was tested simultaneously in equal amounts in the two media.

The results set forth in these tables show what has been observed with some other slightly polluted waters, namely, a larger number of positive coli identifications with lactose broth than with the bile medium. In examining such waters as the one here dealt with, however, it has been argued that only those *B. coli* cells that have

[1] *Jour. Med. Research*, 1912, 20, p. 217.

been in the water a long time are unable to grow in bile media and that the bile-resistant cells indicate "recent or fresh contamination." If this is the case, fresh sewage should contain a relatively

TABLE 2.

LAKE MICHIGAN WATER (LABORATORY TAP).

5 c.c. Samples.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE BROTH		
	No. of Samples	No. Showing *B. coli*	Percentage	No. of Samples	No. Showing *B. coli*	Percentage
No gas	16* (15)	0	0	24	0	0
Less than 10 per cent	0* (1)	0 (1)	0 (100)	0	0	0
10–20 per cent	5	4	80	3	1	33
Over 20 per cent	19	12	63	13	11	85
Total	40	16 (17)	40 (42)	40	12	30

* In one case a fermentation tube showing no gas in 48 hours showed 5 per cent of gas in 72 hours and *B. coli* was later isolated from this tube. The change in figures caused by this is indicated by the figures in parentheses underneath the regular 48-hour records.

The summary was made from 48-hour records of the lactose broth tubes and from 72-hours records of the lactose bile tubes.

In this series, tubes of liver broth (Jackson and Muer, *Jour. Infect. Dis.*, 1911, 8, p. 289) were also inoculated (40 samples). The percentage yielding *B. coli* was about the same as with lactose broth, viz., 37.

TABLE 3.

LAKE MICHIGAN WATER (LABORATORY TAP).

1 c.c. Sample.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE BROTH		
	No. of Samples	No. Showing *B. coli*	Percentage	No. of Samples	No. Showing *B. coli*	Percentage
No gas	72	0	0	100	0	0
Less than 10 per cent	23*	6*	26	6	1	16
10–20 per cent	17	7	41	9	3	33
Over 20 per cent	38	33	87	35	29	83
Total	150	46	31	150	33	22

* In one experiment, a lactose fermentation tube containing 5 per cent of gas in 24 hours was broken so that the 48-hour gas production could not be determined. A plate had been poured before the accident and *B. coli* was later isolated from this plate.

A parallel series of 90 tubes of lactose broth and the same number of liver broth tubes gave 32 per cent of *B. coli* isolations for the former, 27 per cent for the latter.

The summary is based on records taken after lactose fermentation tubes had incubated *48 hours* and bile tubes *72 hours* or longer.

small proportion of *B. coli* cells refusing to grow in bile media. The following table (Table 4) gives the results obtained by examination of 70 1:100,000 c.c. samples of fresh sewage and shows that

here also lactose bile fails to indicate the presence of *B. coli* under conditions where the cells are presumably recently derived from the human body and therefore indicative of recent contamination.

TABLE 4.

SEWAGE FROM THIRTY-NINTH ST. PUMPING STATION, CHICAGO.

AMOUNT OF GAS IN FERMENTATION TUBES	IN LACTOSE BROTH			IN LACTOSE BILE		
	No. of Samples	No. Showing *B. coli*	Percentage	No. of Samples	No. Showing *B. coli*	Percentage
No gas	22	0	0	52	0	0
Less than 10 per cent	3	0	0	0	0	0
10–20 per cent	4	1	25	1	1	100
Over 20 per cent	41	34	83	17	15	88
Total	70	35	50	70	16	23

In all cases recorded above, 1 c.c. of a 1:100,000 dilution of the sewage was inoculated into each fermentation tube. An equal number of lactose broth and lactose bile tubes were made of each sample of sewage. The summary was made on the basis of 48-hour records of lactose broth and 72-hour records of lactose bile.

A parallel series of 20 tubes of lactose broth and liver broth gave 35 per cent of coli isolation for each medium.

It cannot of course be assumed out of hand that lactose broth reveals the presence of all viable cells of *B. coli*. I have elsewhere called attention[1] to the advantage of direct plating on Endo medium for rapid isolation of *B. coli* from certain kinds of water. In the course of this work, it was found that as many colon bacilli grew from pure cultures plated on Endo medium as on plain agar.[2]

TABLE 5.

COLONY COUNT (24 HOURS AT 37° C.).

Strain		Plain Agar	Endo Medium‡
Fecal strains transferred on nutrient agar at 48-hr. intervals	1 (208th transfer)	346	363
	2* (11th ")	19	20
Suspension in flasks of sterile tap water†	3	24	20+
	4	267	324
	5	137	156
Freshly isolated from feces	6	897	735

* Water suspension kept 81 days at room temperature before plating.

† Water suspensions kept 1 year at room temperature before plating.
These results seem within the range of counts often obtained with duplicate samples.

‡ The Endo medium used in this comparison was prepared in the following way: 1,000 c.c. sugar-free broth to which 1 per cent Witte's peptone and 20 gms. of agar have been added. Dissolve in autoclav. Make 0.5 per cent acid with sodium carbonate. Add 10 gms. of lactose and dissolve. Then add 7 c.c. of a saturated alcoholic solution of fuchsin. Decolorize with 16–25 c.c. of a 10 per cent solution of sodium sulfite. A thin plate of the medium, when cool, should be a very pale pink. When sufficiently decolorized, filter, tube, and sterilize for 3 min. at 15 pounds pressure in the autoclav.

[1] E. O. Jordan, "The Bacterial Examination of Water", *Proc. 15, International Congress of Hygiene*, 1912.

[2] Kinyoun's experience seems to have been different from this, since the Endo medium prepared by him inhibited colon bacilli (*Am. Jour. Pub. Health*, 1912, 2, p. 979).

If, as these results indicate, the Endo medium that I have used exerts little or no inhibitive power upon colon bacilli, a comparison of the results with this medium and with lactose broth should be of interest. I have made a few observations in this direction, but a more extended series would perhaps be desirable. The results as far as they go show equal efficiency in the two methods (Table 6). Table 7 shows in another way the suppression of *B. coli* cells by bile. Suspension of pure cultures of *B. coli* in flasks of sterilized water were inoculated in appropriate dilution and in strictly parallel series into the ordinary lactose broth and lactose bile fermentation tubes.

TABLE 6.

PROPORTION OF *B. coli* ISOLATED FROM LACTOSE BROTH AND ENDO PLATES.

TOTAL QUANTITY EXAMINED	NO. OF *B. coli* ISOLATED FROM	
	Lactose Broth	Endo Plates
100 c.c. (Lake Michigan water)*	39	39
5–10,000 c.c. (sewage)†	28	32

* A summary of 10 experiments in each of which 10 c.c. of the sample was plated on Endo's medium and 10 c.c. inoculated into lactose broth fermentation tubes. All colonies at all resembling *B. coli* were picked from the Endo plates, and all gas-producing tubes of lactose broth were plated out in the manner above described (p. 000?).

† A summary of 5 experiments, in each of which 1–10,000 c.c. of the sample was plated on Endo's medium and 1–100,000 c.c. inoculated into each of 10 lactose broth fermentation tubes, making the total amount of the sample the same in both cases.

TABLE 7.

WATER SUSPENSIONS* OF PURE CULTURES OF *B. coli* IN LACTOSE BILE AND LACTOSE BROTH.

Amount of Gas in Fermentation Tube	In Lactose Broth†	In Lactose Bile†
No gas	12	22
More than 20 per cent	18	8
Total number of samples	30	30

† Readings made after 48 hours at 37° C.

* Kept for 102 days at room temperature.

The results as given in the table show about the same degree of inhibition as appears in the experiment with sewage (Table 4).

These observations show that bile inhibits at least from one-third to one-half of the viable cells of *B. coli* and sometimes a much larger proportion; that freshly isolated cultures are inhibited in at least the same degree as those under long cultivation or those

subjected to prolonged sojourn in water; that there is no evidence that *B. coli* cells that are unable to grow on bile medium are any more "attenuated" or less "vigorous," biologically, than their fellows, and that with some care in making dilutions and replating if necessary *B. coli* will be isolated in a larger proportion of cases from lactose broth fermentation tubes than from lactose bile. These facts may not modify the advantages claimed for bile media for presumptive tests, but they do demonstrate that bile is an inhibiting substance for *B. coli* as for other microorganisms and that its use always involves the suppression of a certain number of viable cells. The cells that are suppressed cannot be assumed to be any less significant in the interpretation of sanitary water analyses than the cells actually surviving the passage through bile.